ON THE DEVELOPMENT OF ECTOPIC EYES IN *DROSOPHILA MELANOGASTER* PRODUCED BY THE MUTATION EXTRA EYE (*EE*)

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ABSTRACT

The mutation *ee* often produces an ectopic eye on the vertex that is a mirror image partial duplication of the normal eye on the ipsilateral side of the head. The pattern of the duplication and a clonal analysis by mitotic recombination indicate that the duplications are of dorsal eye and orbital structures. Large ectopic eyes (more than 100 ommatidia) and their surrounding bristles may be produced without cuticular deficiencies. The penetrance of *ee* is temperature dependent with penetrance higher (72%) at 25° and 29° than at 19° (43%). Temperature shift experiments show two temperature-sensitive periods: one at midembryogenesis, the other at mid-first larval instar. Microscopic examination of *ee* late-second and third instar imaginal cephalic discs show no indication of growth of the extra tissue needed to produce the duplication until after mid-third instar. This was confirmed by cell counts of *ee* and wild-type discs. There is no evidence of differential cell death in the two types of discs at this stage, although much earlier cell death is postulated. Tests for cell autonomy of the mutation by the production of morphogenetic clones suggest nonautonomy. Formation of pattern duplications by mutant genes is discussed in terms of cell death that eliminates whole developmental compartments, restricted cell death that occurs within a compartment, extensive cell death within a compartment and proliferative growth unassociated with cell lethality.

MARCEY and STARK (1985) provide evidence that the ectopic ommatidia produced on the dorsal head by the incompletely penetrant *ee* (extra eye) mutation are in their gross and fine structure approximately identical with normal ones. The axons from the photoreceptors of these ectopic eyes only rarely reach the first optic ganglion, but this is the sole important difference between extra and normal eyes. It is apparent that either this mutant is able to redirect the developmental pathway of cells that normally give rise to dorsal head cuticle or, alternatively, to disrupt the field of positional information in such cells with the result that duplicated ommatidia are formed. The normal compound eyes in extra-eye-bearing flies are of standard size and shape (786.8 ± 22.3 ommatidia, $N = 6$).

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To understand how such a diversion in the developmental program of the dorsal head is brought about, we have studied (1) the cuticular pattern of the duplicated ommatidia and their associated orbital chaetae, (2) the cell lineage relationship between the ectopic eye with its orbit and the ipsilateral normal eye, (3) the developmental time at which decisions are made to produce ectopic eyes, (4) the changes in morphogenesis of the *ee* cephalic imaginal disc as compared with a wild-type disc and (5) the cellular autonomy of *ee* tissue insofar as its expression of ectopic ommatidia.

As a result of these studies, we suggest that, even in those cases in which the ectopic eye duplication is unaccompanied by any large tissue deficiency, an extra eye may result from duplication of positional information within the dorsal compartment of the head. Some of the events that set up this duplication of information occur in early development, although manifestation of extra-eye morphogenesis in the imaginal disc is not evident until after the middle of the third larval instar.

MATERIALS AND METHODS

The extra-eye mutation was found by WILLIAM AVERHOFF upon inbreeding offspring of an inseminated female he collected from an Austin, Texas, population. He kindly sent the mutant strain to us for study. The locus of *ee* is on chromosome *2L* between *dp* and *spd* (D. MARCEY, unpublished results).

Clonal analysis: Mitotic recombination was induced by 1200 r of ^{60}Co γ rays administered in about 7 min to larvae 48–56 hr after egg laying. The clones were detected in Minute females from the cross *M(1)o/FM6*; *ee* ♀♀ × *y w* f^{36}; *ee* ♂♂. There is about a 38-hr delay in puparium formation in *M(1)o* flies as compared with wild type (FERRUS 1975); thus, the larvae irradiated were in the latter half of the first instar.

Morphogenetic clones: Mitotic recombination clones were induced by 1000 r of ^{60}Co γ rays administered at about 90 r/min to embryos or larvae of three ages: 0–31, 27–51 and 51–75 hr. The individuals treated were F_1's from crosses of the type *y/y*; $sc^{19}(y^+)$ *M(2)z/CyO* ♀♀ × *y w/y*$^+$*Y*; *ee b pr/ee b pr* ♂♂. F_1 females of the genotype *y/y w*; $sc^{19}(y^+)$ *M(2)z/ee b pr* were scored for clones of the genotype *y/y w*; M^+ *ee*/M^+ *ee* produced by mitotic recombination between *ee* and the centromere. (The y^+ insertion of sc^{19}, *M(2)z* and *ee* are all closely linked.) These clones are recognized by the *y* M^+ bristles produced in the background of Minute bristles with wild-type pigmentation. See LINDSLEY and GRELL (1968) for explanation of genetic symbols and D. MARCEY (unpublished results) for a genetic description of *ee* and its enhancers.

RESULTS

The pattern duplication nature of extra eyes: Individuals homozygous for *ee* display various degrees of cephalic pattern duplication and deficiency, some of which are shown in Figure 1. Some show well-formed, supernumerary compound eyes with surrounding orbital structures (Figure 1A). The duplicated structures are in mirror image symmetry with their normal counterparts, and there is no accompanying deficiency of cuticular structures. Note the similarity of this pattern in Figure 1A with that portrayed in Figure 2A. Figure 1B illustrates a minor duplication-deficiency pattern: an extra ocellus (O) but a missing (square) postvertical (PV) bristle. The most common cuticular deficiency observed is deletion of some or all of the occipital (OCP) bristles as in Figure 1C. In other cases this deletion is accompanied by the appearance of

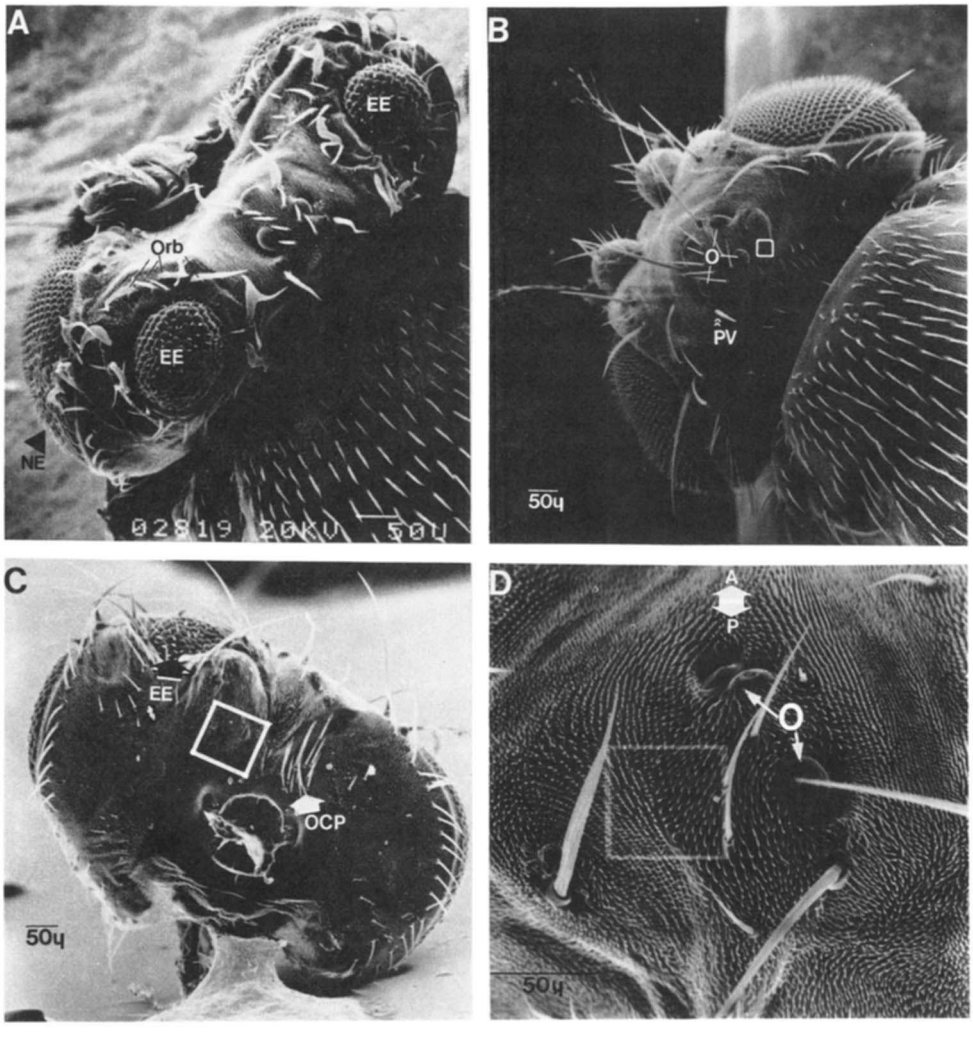

FIGURE 1.—Examples of the extent of variation in the *ee* phenotype. A, *y w* f^{36a}; *ee*. B, C, D, *b pr ee*. EE = extra eye; NE = normal eye; Orb = orbital bristles; PV = postvertical bristles; O = ocelli; OCP = occipital bristles; squares = areas where structures are absent, A = anterior; P = posterior.

occipitals on the dorsal head (see Figure 9). Figure 1D shows a small pattern shift—the left ocellus is missing but an extra anterior ocellus is present.

The appearance of dorsal and posterior head deficiencies are positively correlated with the expression of lateral head pattern duplications. For example, the pattern duplication that forms an ectopic eye with its surrounding bristles is often accompanied by a deficiency of occipital and/or postvertical bristles. The data presented in Table 1 indicate that this correlation holds for strains with three different *ee* penetrances. For example, these data show that flies with both ommatidial duplication and occipital deficiency occur with a much

TABLE 1

Frequency of cephalic landmarks deficient or duplicated in several ee *lines*

	Lateral				Dorsoposterior		
Lines	Eye	Orb	Ver	Po	PVer	Ocp	*N*
Iso-HP	0/0.50	0/0.50	0/0.48	0/0.55	0.53/0	0.57/0	56
Iso-1A	0/0.34	0/0.28	0/0.32	0/0.32	0.47/0	0.39/0	38
Iso-1B	0/0.01	0/0.01	0/0.01	0/0.01	0.02/0.01	0.01/0	98

Results are expressed as frequency deficient/frequency duplicated. Eye, ommatidia; Orb, orbital bristles; Ver, vertical bristles, Po, postorbital bristles, PVer, postvertical bristles; Ocp, occipital bristles, *N*, number of half-heads scored.

higher frequency (0.48 for the Iso-HP line and 0.26 for Iso-IA) than one would expect if their co-occurrence were independent of one another ($0.50 \times 0.57 = 0.28$ for Iso-HP, and $0.34 \times 0.39 = 0.13$ for Iso-IA).

However, quite large pattern duplications with more than 100 ommatidia sometimes occur without any cuticular deficiency. If one calls these cases exceptions, that in no way explains their occurrence, and we seek explanations in developmental terms of each case of ommatidial duplication. We emphasize these examples because (1) they are morphologically simpler than the more usual duplication-deficiency phenotype and (2) they are not easily accommodated under the current models of tissue regulation (see our comments on convergent triplications in DISCUSSION). With this in mind, we selected 26 cases in which a large ectopic eye was present between the ocelli and the ipsilateral normal eye, but the ocelli were normal as were the ocellar, postvertical and interocellar bristles. Thus, there was no obvious cuticular deficiency anywhere on the head except that there was often a decrease in the number of occipital bristles, but this was not accompanied by any cuticle deformity.

In 23 cases among the 26, one or more of the orbital bristles were duplicated in mirror image symmetry one or more times. The inner and outer vertical bristles were duplicated 19 and 22 cases, respectively, out of the 26. These duplicated bristles were between the extra-eye and the ocellar region. In addition, there was a secondary duplication of the outer vertical bristle alongside the normal outer vertical in more than half the cases. In each case a number of postorbital bristles were present around the posterior border of the extra eye. The average number of occipital bristles on the side ipsilateral to the ectopic eye was 6.7, whereas the average number on the contralateral normal side was 10.0.

A typical case of pattern duplication in these 26 flies is presented in Figure 2A. Figure 2B portrays one of the six cases among the 26 in which there was a further mirror image duplication of the orbital bristles. An important thing to note from these figures is that it is only the bristles on the dorsal part of the orbit (orbitals and verticals) that are duplicated around the ectopic ommatidia. Therefore, it seems reasonable to conclude that the postorbital bristles accompanying the extra eye are dorsal postorbitals, and that, if one can legitimately speak of "dorsal" and "ventral" ommatidia, the ommatidia in the ec-

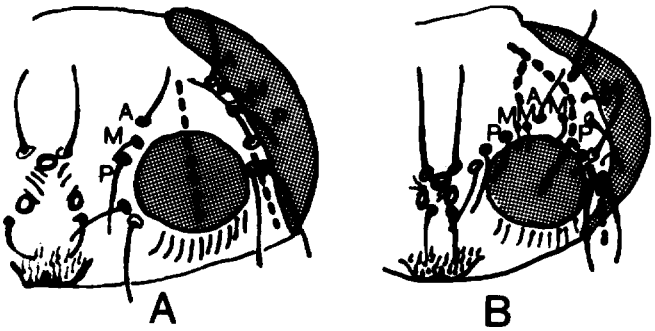

FIGURE 2.—Sketches of typical ectopic eyes without cuticular deficiency. A, Note mirror image duplication (dotted lines mark axes of symmetry) of orbital, vertical and postorbital bristles as well as an additional duplication of the postvertical bristle. B, Pattern triplication of orbital bristles around ectopic eye. A = anterior; M = medial; P = posterior orbital bristle.

topic eye are most likely "dorsal." In other words, these duplications are like other pattern duplications (BRYANT 1978) in that the duplication causes no abrupt discontinuity in the normal pattern of differentiation. As one goes from the dorsal normal eye through the duplication to the normal medial ocellar region, one does not see ventral regions directly in contact with dorsal regions, but rather there is a line of mirror image duplication running through the middle of the ectopic eye. Of course, ventral regions might have been produced without positional discontinuity by producing a complete double ectopic eye; *i.e.*, one might have observed proceeding medially from the lateral normal eye: dorsal ectopic eye, ventral ectopic eye, another ventral ectopic eye, another dorsal ectopic eye and finally the normal ocellar region. Such a triplication should be accompanied—at least in some cases—by ventral orbital chaetae, such as vibrissae or genal bristles, but none was observed. Thus, it seems reasonable to conclude that some large extra eyes are duplications or triplications of only dorsal head tissue.

Clonal relationship between extra eyes and other head tissues: Further evidence on the dorsality of the ectopic eyes comes from experiments in which mitotic recombination clones were induced by γ rays in *y w f/M(1)o; ee* flies. The large *y w f* M^+/*y w f* M^+ clones (MORATA and RIPOLL 1975) so produced form colorless ommatidia in eye tissue and yellow forked bristles and forked trichomes in the remainder of the head. Flies picked for analysis were limited to those with extra ommatidia in which there was a white clone in either the normal eye, or the extra eye, or both. Eighty-one head sides had clones that fulfilled these criteria. The data on these clones are recorded in Table 2, and a random sample of 20 of these are portrayed in Figure 3. Most clones in the normal eye include the orbit, and clones in the extra eye often include its orbit as well (Figure 3f, g, k and q). You will note from Table 2 that the clones in the normal eye are almost all (54 of 58) restricted to either the dorsal or ventral half of the eye, showing the early developmental compartmentalization of the head previously reported (BAKER 1978a; CAMPOS-ORTEGA and WAITZ 1978). Two of the exceptions can be seen in Figure 3b and i. Of the

TABLE 2

Clonal relationship between normal and ectopic ommatidia

No. of cases of clones		Restricted to normal ommatidia		Restricted to ectopic ommatidia		Includes ectopic ommatidia		Includes normal ommatidia	
		Yes	No	Yes	No	Yes	No	Yes	No
In normal ommatidia									
Dorsal	32	9	23			7	16		
Ventral	22	12	10			0	10		
Both	4	0	4			0	4		
Total	58	21	37			7	30		
In ectopic ommatidia									
Total	23			1	22			7	15

clones in the dorsal normal eye, 23 of 32 cross the eye-orbit border and seven of these include extra-eye ommatidia. Reciprocally, clones that include extra-eye ommatidia cross into its orbit in 22 of 23 cases and, as previously noted, seven of these include the normal eye. It is obvious that at the developmental time in which the clones were induced (the latter half of the first larval instar), there are cells present that can be the progenitors of both the normal eye and the ectopic duplication; however, this clonal relation is not obligatory since it is also true that the duplicated bristles around the ectopic eye may belong to a different clone than their mirror image normal counterparts. (Figure 4).

From these data and a perusal of Figure 3, one concludes that the ectopic eyes are clonally related to tissue in the head vertex and to dorsal eye. We had previously shown (BAKER 1978a) that dorsal eye and vertex are in the same developmental compartment at this stage (first instar). Our conclusion that the ectopic eyes are composed of dorsal tissue is supported by our observation (data not presented) that we are not able to perceive a line of clonal restriction in the ectopic eyes while, at this time, the dorsal-ventral restriction line is readily apparent in normal eyes.

It should be noted that an equator (a line separating ommatidia of opposite rhabdomeric spin) forms in these large ectopic eyes (MARCEY and STARK 1985). Since the ectopic eye is probably composed of dorsal tissue, the formation of an equator may not be due to the apposition of dorsal and ventral tissue. Although in normal eyes the equator is in the general region of the boundary between the dorsal and ventral compartments, it was shown previously that it does not follow strictly the compartment border (READY, HANSON and BENZER 1976; CAMPOS-ORTEGA and WAITZ 1978).

Temperature sensitivity of extra-eye expression: One of the classical ways of inferring a time of gene expression is to use a demonstrated temperature effect on expression and determine whether there are precise temperature-sensitive periods (TSPs) during development for this effect. Flies of the Iso-HP strain (see Table 1) were allowed to mate, then transferred to egg-laying food for 4

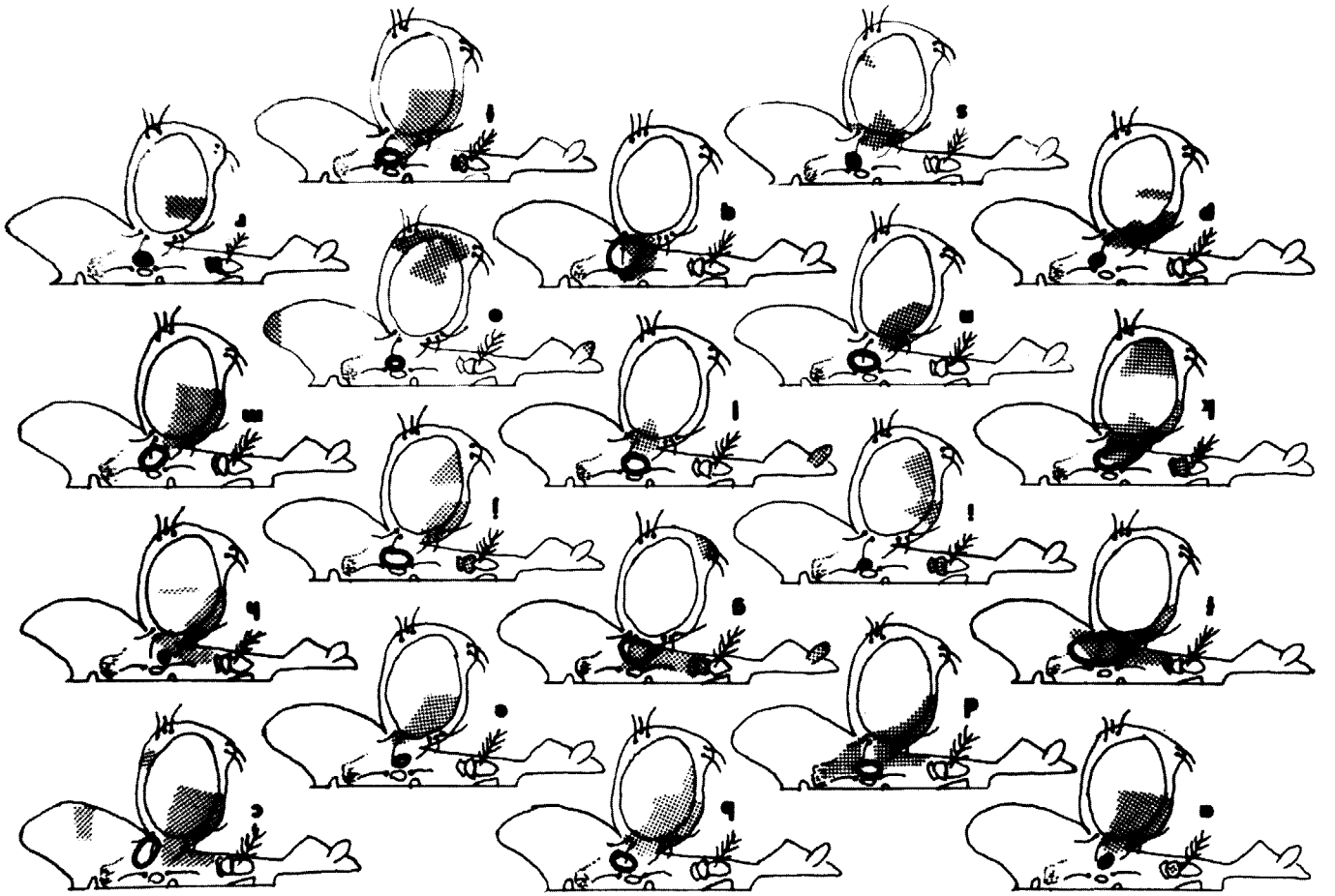

FIGURE 3.—A random sample of 20 of 81 clones induced in *y w f/M(1)o; ee/ee* flies with ectopic ommatidia. The homozygous *y m f* M^+; *ee* clones are shown as stippled areas, and the ectopic eyes are outlined by a heavy line. By cutting out one of these two-dimensional figures and folding to connect the tissue along the anterior and posterior orbital regions, a reasonable three-dimensional facsimile of a half-head will be obtained.

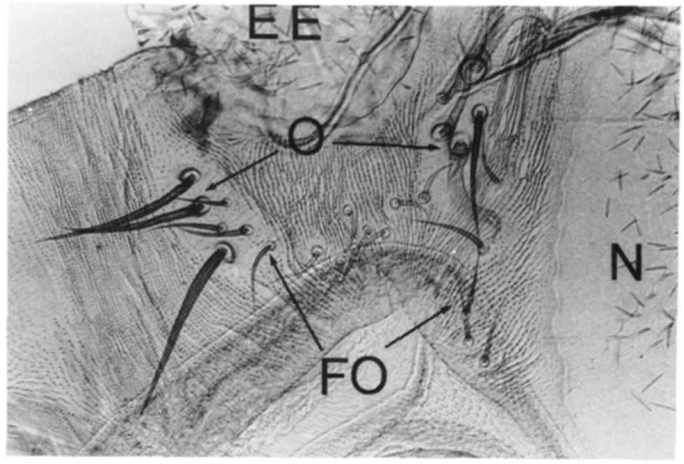

FIGURE 4.—Mitotic recombination clones and mirror image duplication. Note that orbital (O) and frontorbital (FO) bristles around the extra eye (EE) in this *y m f/M(1)o; ee/ee* fly are wild type, but these bristles are *y f* around the normal (N) eye, indicating that in this case the duplicated and normal bristles arose from different progenitor cells.

TABLE 3

Temperature effect on extra-eye expression

		Frequency of flies with different phenotypes		
Temperature	*N*	Wild type	Ectopic facets	Head abnormalities but no ectopic facets
19°	986	0.573	0.303	0.124
25°	1407	0.296	0.617	0.087
29°	557	0.208	0.654	0.084

hr at room temperature and, finally, the adults were removed and the eggs transferred to incubators maintained at 19°, 25° and 29° for the remainder of the life cycle. The resulting imagoes were scored for ectopic ommatidia as well as any duplication or deficiency of cuticular structures on the vertex. The data of Table 3 clearly indicate that the penetrance of ectopic ommatidia as well as cuticular duplications or deficiencies is about twice as high at 25° and 29° as it is at 19°.

Temperature shift experiments were then undertaken to determine whether discrete ontogenetic periods are sensitive to temperature with respect to *ee* penetrance. An outline of the experimental procedure and the results obtained are presented in Figure 5. It is obvious that there is a TSP very early in development because shifts in temperature after this period do not alter significantly the penetrance from that observed by continued presence in the preshift temperature (Figure 5). Of course, development proceeds more slowly at 19° than at 25°; therefore, one must translate the lower curve to the left if one wishes to compare the penetrance at comparable biological ages. The results of such a translation suggest even more strongly that the TSP occurs during the embryo or first larval instar.

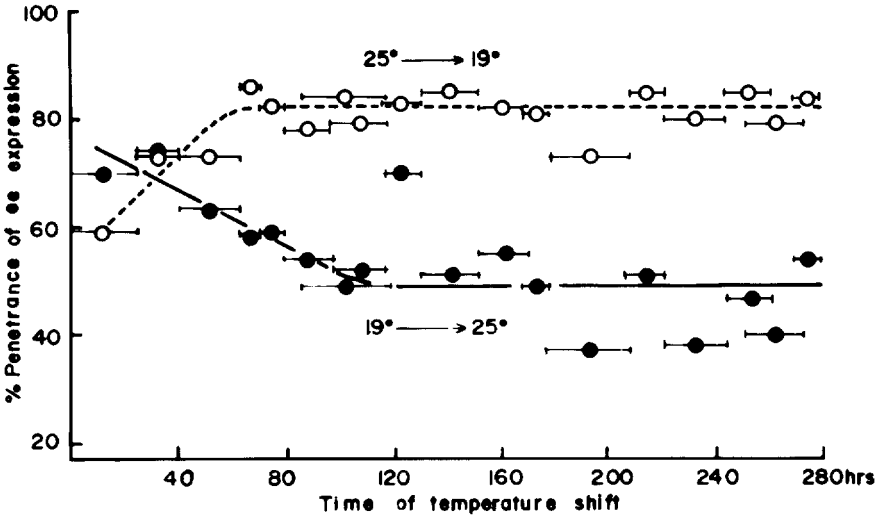

FIGURE 5.—Effect of temperature shifts through larval and pupal life on penetrance of *ee* expression. "Expression" includes ectopic ommatidia and/or cuticular deficiencies or duplications on vertex. Flies from the enhancer *ee* strain were mated at room temperature for 24 hr, then transferred to egg-laying food and placed in an incubator at the preshift temperature where they remained for a period of time indicated by the length of the horizontal line drawn for each data point. At the end of the egg-laying period, the parents were removed and the bottle with eggs was replaced in that incubator until the time at which the temperature shift was made. O, shift from 25° to 19°, ●, shift from 19° to 25°.

To localize more precisely this TSP of penetrance sensitivity, experiments were undertaken in which 13 shifts of temperature in both directions were made at 5-hr intervals during the first 62 hr of development. These data are presented in Figure 6. It is important to note that the two curves have been translated so that a direct comparison of penetrance can be made between stages of comparable developmental age. The two major changes in shape of these curves (arrows) indicate two TSPs for *ee* penetrance, one in midembryogenesis and the other in the first half of the first larval instar. Because *ee* penetrance is also profoundly influenced by enhancer mutations (D. MARCEY, unpublished results), it is not clear whether these TSPs influence the primary *ee* mutation or its enhancers. For technical reasons, temperature sensitivity has not been assayed in a completely enhancer-free line. However, it is apparent that events crucial to the expression of the *ee* phenotype in an individual fly occur during these periods.

Morphogenesis of ee *imaginal cephalic discs*: Ectopic eyes formed on the vertex often involve the addition of a large amount of highly differentiated cuticle to the head, and this should be reflected in abnormal morphogenesis of the cephalic disc. Presented in Figure 7 are a series of late third instar discs from a highly penetrant *ee b pr* strain showing various degrees of ectopic growth in the region of the disc that will form dorsal head imaginal structures. The disc shown in Figure 7E is particularly striking since it has a very large additional eye field. If this larva had been allowed to metamorphose and become an adult, one presumes that a huge ectopic eye would have been produced. To determine when such a shift in morphogenesis first becomes apparent and to

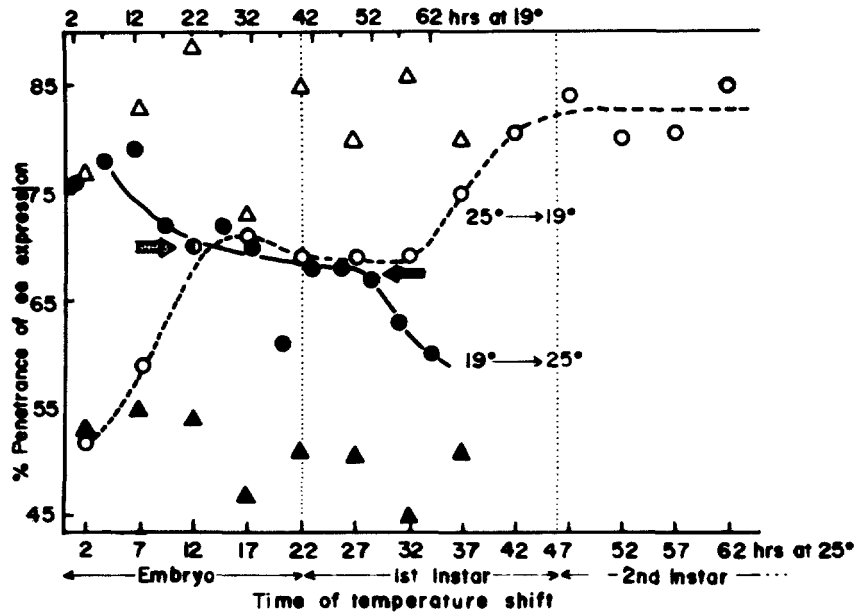

FIGURE 6.—Effect on *ee* penetrance of temperature shifts during embryonic and first larval instar stages. Protocol same as in Figure 5 except that eggs were laid only over a 5-hr period on filter paper soaked with a live yeast solution to which a drop or two of 45% acetic acid was added. The filter papers with eggs were then placed on food in bottles preincubated at the preshift temperature, which ensured rapid equilibration of eggs to the proper temperature. For each data point (time of temperature shift) four groups of flies and their resulting eggs were used: 25° preshift, 25° postshift control (△); 25° preshift, 19° postshift (○); 19° preshift, 25° postshift (●); 19° preshift, 19° postshift control (▲). The data points for the two groups with a preshift temperature of 19° have been placed on a time axis that has been translated in order to produce that same developmental stage at the time of temperature shift. The 19° and 25° controls did not accompany the shifts done after 32.5 hr. Arrows indicate the two TSPs.

determine whether this is accompanied by cell death, discs from larvae of different ages were dissected very carefully in 0.4% neutral red, stained for at least 4 min in 0.25% trypan blue (DERENZIS and SCHECTMAN 1973), and examined immediately under a compound microscope. About one of every nine discs was damaged in the process of dissection producing a wound of dark blue dead cells on the disc surface (see Figure 7D), and these discs are not included in our analysis. The data derived from the successful dissections are compiled in Table 4. We saw no evidence of cell death during the late-second or third larval instar that is associated with the duplication phenotype. Of course this does not rule out the presence of earlier cell death, since such dead cells could be quickly removed. Also we could detect no extra tissue growth in the part of the *ee* disc that will form dorsal head until after mid-third instar.

To substantiate the latter observation, *ee* and wild-type discs were dissected from larvae at 10-hr intervals beginning midway through the third instar (80 hr) and extending until pupariation. The cells of the discs were dissociated, and their number was determined by using a hemacytometer. A highly penetrant *ee b pr* strain was used—the incidence of ectopic ommatidia per fly was about 40%—but this means that, among the randomly selected *ee* discs to be

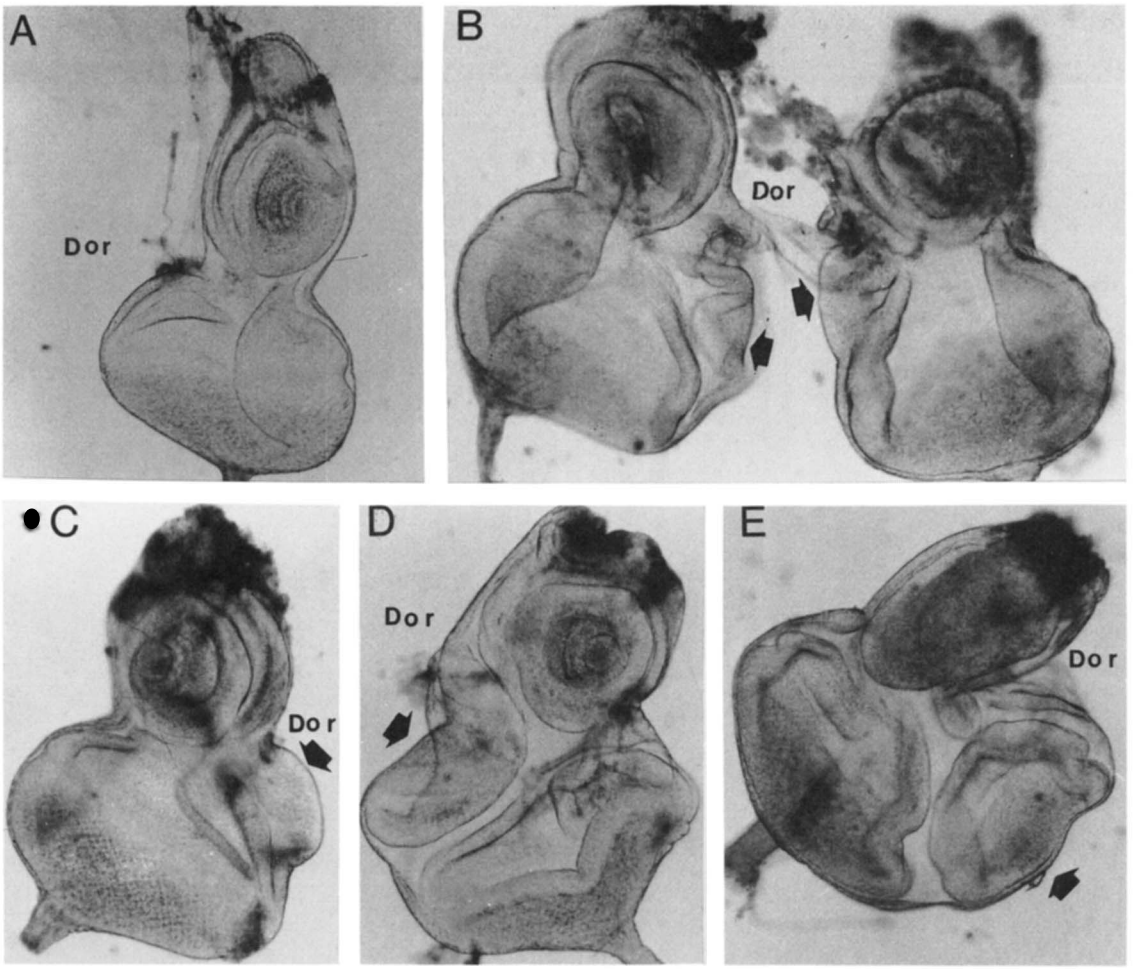

FIG. 7.—The variation in expression of *ee* in the cephalic imaginal discs. Discs of late third instar wild-type (A) and *ee* (B–E) larvae were dissected in neutral red and then stained in trypan blue to indicate any cell death. Note in E the large developing supernumerary ommatidial field produced in the region of the disc that would normally form dorsal (Dor) head. Arrows indicate the region of ectopic growth. Note darkly staining spot in the antennal region of disc D caused by damage during dissection.

TABLE 4

Cell death and morphology in ee *imaginal cephalic discs*

Developmental stage	Oregon R strain	*ee b pr* strain morphology	
		Normal	Mutant
Late 2nd to mid-3rd instar			
No. discs without dead cells	11	57	0
No. discs with some dead cells	1	8	0
Late 3rd instar			
No. discs without dead cells	23	58	44
No. discs with some dead cells	2	5	5

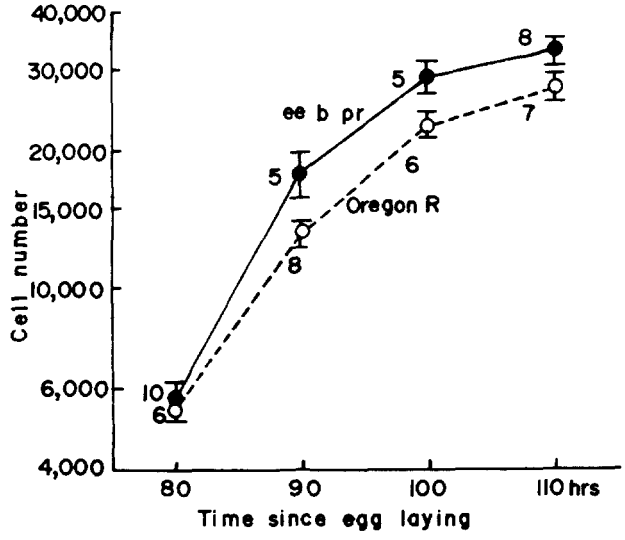

FIGURE 8.—Number of cells in extra-eye cephalic discs (*ee b pr*, high-penetrant line) as compared with wild type (Oregon R) taken from larvae in the last half of the third instar. The number of discs dissociated and counted is given beside each point; the bars give the standard error of the mean cell count. The collection of the larvae, their culture and the dissociation of the discs by citric acid were all done using the protocol of MARTIN (1982), as was the cell counting using a hemacytometer.

dissociated, about 20% would have the additional disc growth necessary for pattern duplication. Therefore, the difference in cell number between *ee* and wild-type discs is a minimum figure. The data in Figure 8 substantiate our cursory visual observations: no demonstrable difference in cell number at 80 hr, but a consistent larger number of cells in the *ee* as compared with wild-type discs from then on until pupation.

The temperature shift experiments clearly show that during late embryogenesis and early first instar decisions are made in an individual *ee* fly influencing the probability that it will form an ectopic eye. Clonal analysis done by mitotic recombination indicates that at the end of the first instar the normal and ectopic eye can have a common ancestral cell. Therefore, the early decisions promulgating the extra cell divisions necessary to form an ectopic eye are

TABLE 5

Frequency of morphogenetic clones of M^+ ee *tissue induced in* y w/y; sc^{19} M(2)z/ee *females*

Time of irradiation (hr)	No. of females scored	No. of females with clones at least in ocellar region
0–24	2736	7[a]
27–51	2871	33 (1)
51–75	3692	44 (2)
Total	9245	84 (3)

[a] In one experiment four of 2461 flies had clones, but the radiation dose was only 500 r; however, in all other experiments the dose was 1000 r.

In parentheses are the numbers of flies showing ectopic ommatidia within the clone.

either not clonally inherited or, alternatively, only the cellular progenitors of the vertex respond to the clonally inherited early decisions. Finally, it appears either that the cells that give rise to the ectopic eye do not divide continually during disc development as do normal eyes (BECKER 1957), but rather initiate the necessary cell divisions only in the last half of the third instar, or that the ectopic eye progenitors are very much fewer in number than the normal eye precursors and the former continue cell division after the latter have stopped.

Cellular autonomy of ee *tissue*: We tried to gain evidence on whether the pattern duplication of ectopic eyes is a cellular property or a developmental field property by forming morphogenetic clones of *ee/ee* tissue in *ee/+* flies (under the conditions of our experiment, *ee* is recessive), but this approach has not been wholly successful. Mitotic recombination was induced in females of the genotype *y/y*: $sc^{19}(y^+)$ *M(2)z/ee* at three developmental times. Since all three of the second chromosome markers are tightly linked, recombination between them and the centromere will produce a clone of *y*; M^+ *ee/y*; M^+ *ee* cells. If cells within this clone form bristles, they will be yellow and have wild-type structure (in contrast, bristles in the remainder of the fly will have wild-type pigmentation and Minute structure), thus marking the presence of a clone irrespective of whether or not ectopic ommatidia are formed. Flies were scored that have a *y* M^+ ocellar and/or postvertical bristle, indicating a clone in the ocellar region. Heads of these flies were removed and processed for examination in a compound microscope to learn in detail the extent of the clone. The number of flies with such clones induced at different developmental times are recorded in Table 5. We were surprised at the rarity of flies with clones in the ocellar region even at later developmental periods. There must be very few cells that are the progenitors of the ocellar region at the developmental times we irradiated. Even more disappointing was the fact that only three flies with ectopic ommatidia were observed among the 84 with clones on the vertex. Since a low frequency was observed in the first set of experiments completed, we resynthesized the strains needed to make the cross, but this time we incorporated genetic enhancers that elevate the penetrance and expression of *ee* (D.

TABLE 6

Size distribution of morphogenetic clones of ee *tissue*

Regions included in clone	*N*	Regions included in clone	*N*
Oc	29[a]	Oc + Ver	2
Oc + Ocp	9[b]	Oc + Orb + Ver	2
Oc + Ver + Ocp + Po	9	Oc + Orb + Ocp	2[c]
Oc + Orb + Ver + Ocp	8	Oc + Ver + Po	2
Oc + Orb	7	Oc + Orb + Po	1
Oc + Orb + Ver + Ocp + Po	7	Oc + Ocp + Po	1
Oc + Ver + Ocp	4	Oc + Orb + Ocp + Po	1
			(Total) 84

Oc, ocellar, interocellar or postvertical bristles; Ocp, occipital bristles; Orb, anterior, medial or posterior orbital bristles; Ver, inner or outer vertical bristles; Po, postorbital bristles. [a,b,c] = A single fly had a clone within which was an ectopic eye with approximately 20, 10, and 31 ommatidia, respectively.

MARCEY, unpublished results). This did not noticeably increase the frequency of ectopic ommatidia among the flies with ocellar clones. The extra-eye expression is clearly associated with the clone formation since only six of 9245 (0.06%) *M* flies had ectopic ommatidia but no yellow clone on the dorsal head (*ee* sometimes shows conditional dominance; D. MARCEY, unpublished results), whereas three of 84 (4%) *M* flies with clones showed extra ommatidia, a 55-fold difference.

The low frequency of expression does not seem to be due to the fact that the clones are small. As shown in Table 6, more than 60% of the clones extend to another distinct head region in addition to the ocellar area. In fact, 25 of the 84 clones are very large and include either four or five of the five main regions of the dorsal and posterior head surface, yet none of these has ectopic ommatidia. A majority of the clones (46 of 84) extend across the dorsal head surface between the normal orbit and the ocelli, the region where ectopic ommatidia are usually found. Perhaps the low frequency of *ee* expression is due to the fact that, as suggested by the temperature shift experiments, decisions to make ectopic ommatidia are made in late embryo and in the first half of the first larval instar, and most of the clones were induced at a later time. Although only eight of the 84 flies with clones had them induced at this early time (first instar), four of the eight clones are rather large but still there is no *ee* expression.

In the three cases in which there are ectopic ommatidia associated with the clone, we were interested to learn whether the duplicated tissue lies totally within the clone (*i.e.*, genotypically, *ee/ee*) or whether there are duplicated structures outside (genotypically, *ee/+*). The question has a bearing on whether the duplications are formed by disruption of the positional information field in the imaginal disc followed by cell division (BRYANT 1978), in which case *ee/+* tissue might contribute cells to the duplication, or the duplication is caused by cellular transformations (as in cell-autonomous homeotic mutants, GARCIA-BELLIDO 1977), in which case one would always expect the duplicated struc-

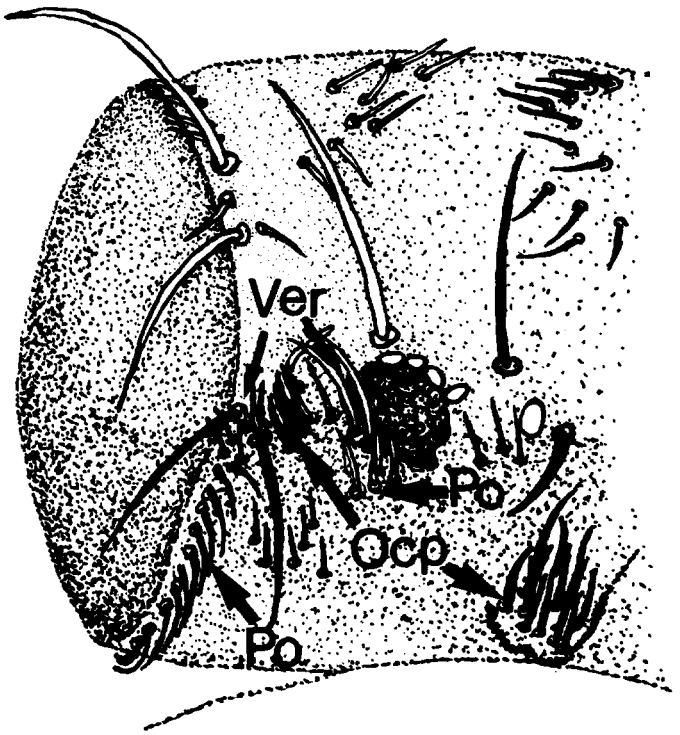

FIGURE 9.—Drawing of head of a *y/y*; $sc^{19}(y^+)$ *M(2)z/ee* female with a *y/y*; M^+ *ee*/M^+ *ee* morphogenetic clone. The ectopic eye contains about 31 ommatidia. The clone—indicated by M^+ yellow bristles (not filled in drawing)—includes frontorbital, frontal, orbital and ocellar bristles as well as some bristles surrounding the ectopic eye which we interpret as verticals (Ver) and postorbitals (Po). There are no occipital bristles (Ocp) in their normal position on side of head ipsilateral to ectopic eye; however, there are occipital bristles on vertex, but they are not within the clone. The ocelli have been duplicated.

tures to be limited to *ee/ee* clones. One of the three cases showed duplicated structures outside the clone, and it is illustrated in Figure 9. This is a large clone that includes all of the frontal and frontoorbital bristles, all three orbital bristles and what appears to be the normal ocellar bristle. Within this clone are about 31 ectopic ommatidia, and lateral to this extra eye are three *y* M^+ bristles that appear to be duplications of the inner and outer verticals, and more posterior are five *y* M^+ bristles that probably are duplications of postorbitals. Note the disarrangement and duplication of ocelli and the absence of any occipital bristles at their normal location. Although this pattern might seem to indicate that the duplicated structures are within the large clone, note the group of bristles between the duplicated vertical bristles and their normal counterpart. Based on morphology these are almost certainly occipital bristles, and they are y^+ *M*. In extra-eye flies we often find a reduction in the number of ipsilateral occipital bristles at their normal site simultaneous with the appearance of a few in the region between the ectopic ommatidia and the vertical bristles. These ectopic occipital bristles are not just "moved" dorsally from

their normal location in the occiput because our mitotic recombination studies (data not presented) show they can be in a different clone from the remaining occipitals in their normal location. Therefore, we suggest that *ee* is not a cell-autonomous mutant. Neither of the other two cases of morphogenetic clones with ommatidia is associated with cuticular deficiencies, and each clone contained all of the duplicated bristles around the ectopic ommatidia.

DISCUSSION

Extra eye: Homeotic transformation or field disruption? There are at least two general types of mechanisms that may be responsible for the pattern duplications produced by mutation: homeotic transformations and disruptions in a field of positional information elaborated in developing imaginal discs caused by cell death or extraneous cell division. Homeotic transformations involve a shift in the determination of one developmental compartment into another, but the positional information field remains the same in the two compartments (GARCIA-BELLIDO 1977). These two general mechanisms may be distinguished by learning whether or not the pattern duplications are always cell autonomous—as they must be if they are of the homeotic type. For this reason we produced by mitotic recombination morphogenetic clones of *ee/ee* tissue in a background of *ee/+* cells. One resulting pattern duplication (pictured in Figure 9) contains some ectopic occipital bristles that are *ee/+* ($y\ M^+$). Since this ectopic location of occipital bristles often accompanies the extra-eye duplication, we consider this evidence for nonautonomy of *ee*. Because homeotic switches should always be cell autonomous, it seems to us that this likely case of nonautonomy makes it improbable that homeotic transformation is responsible for the pattern duplication. Furthermore, in some cases of the extra-eye duplication it does not appear as if anything has been transformed since there are no missing structures, just added tissue. Finally, the eye duplications are intracompartmental events, not transformations between compartments, as is usual with homeotic transformations.

The pattern duplications caused by field disruption are commonly interpreted to be the result of removal of cells—either by surgery or by cell death—from the field, and certain of our results on extra eye are compatible with this interpretation. On the other hand, the cases we observe of large extra eyes with their accompanying dorsal orbital bristles without any sign of deficient tissue suggest that sometimes the field disruption might be caused by extraneous cell division rather than cell death. We shall discuss some theoretical aspects of these two possibilities in turn before relating them to the extra-eye results.

Field disruption by cell death and the possible remedial influence of developmental compartments: Mutations causing cell death can disrupt a field of positional information to produce pattern duplications, and in fact the duplication-deficiency nature of pattern duplications induced by mutant genes has led many investigators (reviewed by GIRTON and BRYANT 1980) to interpret their origin in the same manner as the pattern duplications produced by surgical or radiation-induced extirpation of regions of imaginal discs followed by cellular pro-

liferation of the experimental piece of the disc. BRYANT (1971) and FRENCH, BRYANT and BRYANT (1976) have proposed a model that successfully explains in many cases why the experimental piece of the disc will regenerate the missing structures excised by the operation or why this piece will form a pattern duplication with an accompanying deficiency. Before cell proliferation takes place the experimental disc piece must have acquired positional information such that the positional values of the extirpated region are missing from the experimental piece. Next, the remaining positional values of the experimental piece are "read" and the missing values supplied to the new cells that are formed by cellular proliferation between the cut edges of the experimental piece. If the values in the experimental piece are a minority of those in the entire disc, then the piece will form a duplication-deficiency pattern; if the values are a majority, it will regenerate. It is essential to note that with this mechanism cell proliferation necessary to produce the duplicated tissue must occur *after* the cells in the disc have attained positional values.

This raises the question as to when the positional values that lead to the final pattern of differentiation are specified. In Drosophila the specification of final positional values *within* a developmental compartment of a disc does not occur until most cell division in that disc is completed. This is known because a mature compartment can be composed predominately of the descendants of a single cell in the case of an M^+/M^+ clone growing in a background of M/M^+ cells; yet, this compartment differentiates into perfectly normal tissue [first observed by MORATA and RIPOLL (1975) and by numerous other investigators who have used this technique extensively since that time]. In fact these clones of relatively rapidly dividing cells can be induced from blastoderm through most of the third larval instar and no abnormal differentiation results. For example, M^+/M^+ clones induced in the wing disc at 97–120 hr (the first half of the third instar in these Minute flies) cover 30–50% of the cells in a compartment (GARCIA-BELLIDO, RIPOLL and MORATA, 1976). In other words, up until close to the completion of cell division in the disc all cells in a compartment are equipotent to differentiate into any structure to be formed by the cells of that compartment—at no stage in the ontogeny of a compartment are positional values an inherent property of a cell and its descendants.

The behavior of the M^+/M^+ clones suggests that if cell death occurs early in disc development, and if cellular proliferation is initiated shortly thereafter, then either cells are responding to positional values other than their final ones or else they are responding to some signal other than positional information. We would like to suggest that the cell number within a compartment could serve as a signal. This suggestion is again based on studies of M^+/M^+ clones. In spite of the differential in cell division rate within the developing compartment caused by these clones, the size and shape of the tissue differentiated by that compartment are normal in every respect. Thus, cell division continues in the developing compartment until the proper number of cells are made—the compartment is said to be "filled"; then division stops. This suggests that, if cells die at the proliferative stage during which the compartment is being filled, no abnormal differentiation would ensue because division would con-

tinue until the compartment is filled. We know, in fact, that this is true in certain cases. Every time an M^+/M^+ cell is produced by mitotic recombination in an M/M^+ individual, a sister M/M cell is produced and this cell dies (STERN and TOKUNAGA 1971); yet, the differentiation is normal. Perhaps even more pertinent is the loss through competition of cells of an M/M^+ clone growing in a background of M^+/M^+ cells (MORATA and RIPOLL 1975; BAKER 1978a; SIMPSON 1979; SIMPSON and MORATA 1981). Such loss of a whole clone of cells during maturation of a compartment causes no abnormality in differentiation. We would like to propose that this regulative capacity is the result of *number regulation* rather than by *positional regulation* as long as the event initiating the regulation occurs within a compartment. The number regulation, if complete, will produce a compartment with the proper number of cells to which the final positional values may be assumed.

Although restricted cell lethality within a compartment may not lead to any disturbance of morphogenesis, one can conceive of mutants that produce cell death so extensive and/or so late in the maturity of a compartment that the number-regulated cell divisions within the compartment are not sufficient to produce the normal final number of cells in that compartment at maturity. One might suppose that this smaller set of cells could assume a complete set of final positional values thereby producing a smaller replica of the tissue differentiated by that compartment, but this is not usually observed. Alternatively, one can imagine that there are not sufficient cells to assume all the positional values necessary to specify the structures normally differentiated by the compartment, thus leading to neighboring cells with disparate values. This, in turn, could produce proliferative growth after the attainment of final positional values resulting in regeneration or duplication deficiency in accord with the Bryant-French model. Therefore, incomplete number regulation could lead to positional regulation. It is quite possible that this type of cell death is responsible for many of the pattern duplications and triplications produced by the temperature-sensitive cell lethal *l(1)ts726* (RUSSELL 1974; RUSSELL, GIRTON and MORGAN 1977; CLARK and RUSSELL 1977; POSTLETHWAIT 1978; JÜRGENS and GATEFF 1979; GIRTON and RUSSELL 1980; GIRTON 1981; GIRTON and RUSSELL 1981; GIRTON 1983). RUSSELL (1974) used disc area as a measure of proliferative growth of the disc following a 29° pulse (48 hr) that induced lethal cells in *l(1)ts726*. He found that in both wing and cephalic discs the temperature pulse in the control (not carrying *l(1)ts726*) discs did not affect growth rate, but that the mutant discs did not resume substantial growth until after the control discs ceased growing; however, both control and mutant discs continued growing until their normal size was reached. Thus, it is at least feasible that the growth in these mutant discs necessary to produce duplicated structures resumed only after final positional values had been assumed.

Let us next consider cases of induced cell lethality that are so extensive that *all* cells from a given developing compartment are eliminated. From an operational viewpoint, such extensive lethality would have to be the result of events induced quite early in development when the number of progenitor cells of the compartment is small, since the lethality induced must be such that it can

spread to all progenitors of the compartment. The "spreading" of lethality might, for example, be caused by chromosome breakage in one cell and the initiation of a breakage-fusion-bridge cycle. When the normal proliferative growth of such a disc approaches its end and final positional values are being read, this disc is missing a whole compartment which would lead to zygote lethality or to a large tissue deficiency.

Let us summarize the effects of restricted cell lethality induced at different stages in the ontogeny of an imaginal disc. Induction very early when there are relatively few cells in the disc and the main compartments have not been established can lead to (1) elimination of whole compartments with the ensuing zygote lethality or tissue deletion or (2) such a large numer of lethal cells in a subsequent compartment that the ensuing number regulation is not sufficient to fill the compartment upon maturity. This results in duplication-deficiencies by position regulation. If the restricted (localized to a single compartment) cell lethality is induced after the main compartments are formed (when now there are relatively more cells in a developing compartment), then the number-regulated processes are sufficient to produce normal morphogenesis in spite of cell death. Finally, if the lethality is induced sufficiently late in the compartment maturity, then there is not sufficient time remaining for number regulation to fill the compartment and this cell deficiency can lead to the duplication deficiency phenotype by positional regulation. It is interesting that late restricted cell lethality and very early restricted cell lethality may lead to similar abnormal phenotypes, whereas induction of lethality at an intermediate stage may produce no abnormality at all.

These considerations are supported by the results of POSTLETHWAIT and SCHNEIDERMAN (1973) who observed that cell death produced by ionizing radiation was able to induce pattern duplications of legs and antennae when administered to embryos or to 24-hr first instar larvae that had just hatched from eggs, but absolutely no duplications were observed when irradiation was delivered at 20-hr intervals from 40 to 120 hr after egg laying, a striking but previously unexplained result. Further support for these theoretical considerations is found in the close similarity in appearance between the leg duplications and triplications induced by irradiation at very early stages by POSTLETHWAIT and SCHNEIDERMAN (1973) and the triplicated legs pictured by GIRTON (1983) which were induced by a temperature pulse during the third instar in *l(1)ts726* larvae.

Field disruption by extraneous cell division: Atlhough we know of mutations that cause localized cell death at a particular developmental stage and disrupt the positional field, there is no *a priori* reason for ruling out the existence of mutations that cause a burst of localized proliferative growth at one developmental stage and cause the same effect. If such a localized burst of cell division within a compartment were to occur after normal cell division ceased, one might expect the cells in this area to take on new positional values such that no abrupt discontinuity in values occurs across the area. For example, in a circumferential field of positional values 1, 2, . . . 5, 6, . . . 12, let us assume that a group of new cells are inserted between values 5 and 6. The new cells

could take on values in several ways without causing discontinuity: 1, . . . 3, 4, 5, 4′, 3′, 4′, 5′, 6 . . . 12 and 1 . . . 5, 6′, 7′, 8′, 9′, 8′, 7′, 6, 7, 8, 9, . . . 12, which produce mirror image duplications, or 1 . . . 5, 6′, 7′, 8′, 7′, 6′, 7′, 8′, 7′, 6, 7, 8, . . . 12, which is a mirror image triplication, or 1 . . . 5, 5′, 5′, 6 . . . 12, which is a triplication of a single structure. No tissue deficiency is produced in these cases.

The developmental basis of extra eye: There are five aspects of our results that must be accounted for by any hypothesis of extra-eye formation based on disruption of a field of positional values: (1) the very early TSPs of extra-eye penetrance, (2) the lack of evidence of cell death during the third instar, (3) the extra growth during the last half of the third instar of the *ee* as compared with wild-type discs, (4) a duplication of dorsal head structures including extra ommatidia that is often accompanied by occipital deficiencies, (5) the significant number of cases in which ommatidial and orbital duplication (sometimes triplication of orbital and outer vertical bristles) is not accompanied by any tissue deficiency.

The cephalic disc is determined at the time of blastoderm to produce head structures, and shortly thereafter a dorsal and ventral compartment is established in the eye-head part of the disc (BAKER 1978a). The cephalic disc is not divided into anterior and posterior compartments until middle to late second instar (MORATA and LAWRENCE 1979). At the end of the second instar the anterior dorsal head compartment appears to be further subdivided into additional compartments (BAKER 1978a), one of which includes the dorsal and occipital head regions. Thus, the TSPs for *ee* penetrance occur when the dorsal-ventral compartmentation is being laid down, and restricted cell death produced by the *ee* mutation at that time could lead to such extensive death within the dorsal-posterior head compartment that number regulation is incomplete and positional regulation ensues at the end of the third instar in much the same manner as proposed for the radiation-induced duplications of POSTLETHWAIT and SCHNEIDERMAN (1973). The vertex duplications accompanied by occipital deficiencies observed is the type expected according to the anlage plan of the 96-hr cephalic disc proposed by GATEFF and SCHNEIDERMAN (1975) since the vertex and occiput are adjacent in the plan and would thus be expected to have neighboring positional values. Under such an interpretation one would expect that the dead cells produced at early first instar would be removed long before late-second and third instar when we examined the discs for cell death, and the extra disc growth we observed at the end of the third instar would be the result of positional regulation.

The cases we observe of large extra eyes with no tissue deficiency but with triplicated outer vertical and/or orbital bristles superficially resemble the convergent triplications of leg tarsal segments studied by GIRTON (1981, 1983), where there is one complete leg and two partial tarsal segments produced by incomplete regulative growth. Although GIRTON (1981) observed duplication of both head and leg structures by temperature-pulsing *l(1)ts726* larvae 4–10 hr after the 2/3 instar molt, he apparently observed only triplicated legs. This suggests an inherent difference between cephalic and leg discs insofar as their

capacity to form pattern triplications following cell death. The ingenious modification of the polar coordinate model he has proposed (GIRTON 1981) as a basis for triplication rests almost entirely on the fact that the leg disc is composed of concentric rings of repetitive positional values, to produce, for example, the five tarsal segments. The cephalic disc has no radially repetitive pattern of determination, and furthermore, the structures triplicated on the head (orbital and outer, but not inner, vertical bristles) are located in the anlagen plan at the periphery of the disc (GATEFF and SCHNEIDERMAN 1975; BAKER 1978b); whereas, the distal tarsal segments are most likely to be triplicated and they are determined by cells in the center of the leg disc. These observations suggest that caution should be exercised in any attempt to place the origin of cephalic triplications in the same mold as leg triplications since we know so very little about tissue regulation in the cephalic disc.

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